Comparison of the Activity of Free and Liposomal Amphotericin B In Vitro and in a Model of Systemic and Localized Murine Candidiasis

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Abstract: Because of the toxicity of amphotericin B-desoxycholate (AmB-d) during systemic therapy, less toxic forms of AmB, which promise to have a broader therapeutic index, are under investigation. There is, however, no convincing explanation of how such preparations might be made less toxic yet retain their antifungal efficacy. In this study, the antifungal activity of a less toxic, unilamellar liposomal (1) preparation of AmB (AmBisome), which is commercially available in some countries, was compared with conventional AmB-d in vitro and in models of systemic and localized candidiasis in immunosuppressed mice. Results indicate that 1AmB has four to eight times less antifungal activity than AmB-d in all experimental settings tested. Because 1AmB is significantly less active, the therapeutic index of such preparations must be tested clinically before their use can be recommended solely on the basis of toxicity data

DOI: https://doi.org/10.1093/infdis/169.5.1057

Comparison of the Activity of Free and Liposomal Amphotericin B In Vitro and in a Model of Systemic and Localized Murine Candidiasis

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Because of the toxicity of amphotericin B–desoxycholate (AmB-d) during systemic therapy, less toxic forms of AmB, which promise to have a broader therapeutic index, are under investigation. There is, however, no convincing explanation of how such preparations might be made less toxic yet retain their antifungal efficacy. In this study, the antifungal activity of a less toxic, unilamellar liposomal (l) preparation of AmB (AmBisome), which is commercially available in some countries, was compared with conventional AmB-d in vitro and in models of systemic and localized candidiasis in immunosuppressed mice. Results indicate that lAmB has four to eight times less antifungal activity than AmB-d in all experimental settings tested. Because lAmB is significantly less active, the therapeutic index of such preparations must be tested clinically before their use can be recommended solely on the basis of toxicity data.

More than 30 years after its introduction into clinical practice, amphotericin B (AmB) remains the most important antifungal agent for the treatment of most invasive mycoses [1]. Because of the considerable toxicity of AmB [1–3], which limits its maximal dosage in humans to 1.5 mg/kg/day, less toxic alternatives are sought. Attempts have been made to lower AmB toxicity by entrapping it in liposomes [4–8], complexing it with lipids [9–12], or administering AmB-desoxycholate (AmB-d) in a lipid emulsion [13–16]. However, it is the potential of AmB to preferentially bind to ergosterol rather than to cholesterol that is the key to understanding its fungicidal and toxic activity [17–19]. This chemical property of AmB is not altered by its formulation. Although it has been hypothesized that alternate formulations of AmB might favorably affect its therapeutic index [12, 20, 21], these claims have not been substantiated in clinical studies. New formulations of AmB have, however, been found to be less toxic [8, 9, 13, 22, 23].

In this study, we investigated, in models of experimental murine candidiasis, whether a liposomal preparation of AmB (lAmB), while less toxic, would also be less active than AmB-d. Since hematogenous infections of parenchymatous organs and a secluded infection could differ in their response to therapy with free and lAmB, the therapeutic response was studied in two models: systemic candidiasis after intravenous (iv) infection and localized candidiasis in a subcutaneous pouch [24, 25]. We reduced possible interference of therapeutic effects with host immunity by immunosuppression with cortisone, which prevents elimination of candidae by host defense mechanisms in this model [25].

Received 9 August 1993; revised 22 November 1993.

Presented in part: First annual meeting of the Swiss Society of Infectious Diseases, Geneva, June 1992.

Grant support: Swiss National Science Foundation (32-26411).

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The Journal of Infectious Diseases 1994;169:1057–61

0022-1899/94/6905-0017$01.00

Materials and Methods

Animals. Female ICR mice (Institut für Tierzucht, University of Zurich; 6–8 weeks old; average weight, 25 g) were held in groups of 8 and given free access to food pellets and acidified water.

Organisms. Single lots of *Candida albicans* SD1, [24, 25], NC19, NC31, and SN35 (gift from A. Polak, Hoffmann-La Roche, Basel, Switzerland), isolated from patients with fungemia, were stored at −70°C and propagated overnight at 37°C in 50 mL of tryptic soy broth (TSB; Difco, Detroit). Before use, the isolates were washed three times in 0.9% saline and then diluted in 0.9% saline to desired concentrations. Yeast cells were counted with a hemocytometer, and the inoculum size was verified by quantitative culture of serial dilutions on tryptic soy agar (TSA; Difco).

Immunosuppression. For immunosuppression, mice received 5 mg of cortisone acetate (Sigma, St. Louis) subcutaneously in 0.9% saline 2 days and immediately before challenge [24, 25].

Models of infection. For disseminated infection, 10^4 to 7.5×10^5 yeast cells suspended in 0.5 mL of 0.9% saline were inoculated into the lateral tail vein. For studies of local candidiasis, pneumatized subcutaneous pouches were formed 5 days before challenge as described previously [25]. Briefly, 3–4 mL of air was injected in the back region through a hypodermic needle into the subcutis. To keep pouches pneumatized, air (usually 0.5–1 mL) was reinjected as required during the study. Cysts were challenged with $\sim 10^6$ yeast cells suspended in 0.2 mL of 0.9% saline.

Antifungal regimens. AmB (Fungizone; Bristol-Myers Squibb, Princeton, NJ), consisting of 50 mg of AmB plus 41 mg of desoxycholate, was dissolved in 10 mL of sterile water as a stock solution and then diluted further in 5% dextrose. AmB-d in a dose ≤1 mg (active drug)/kg was given in a single daily injec-

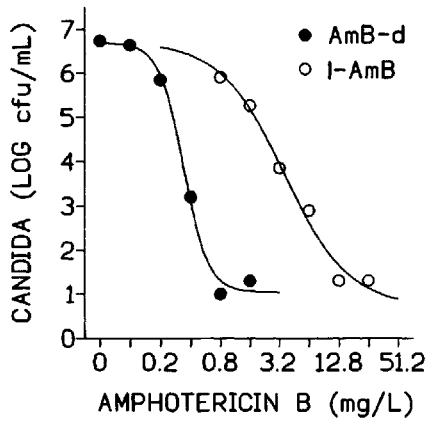

Figure 1. Antifungal activity of amphotericin B–desoxycholate (AmB-d) and liposomal amphotericin B (l-AmB) in vitro: 10^6 cfu/mL *Candida albicans* SD1 was incubated 24 h in medium with graded concentrations of AmB-d and l-AmB before quantification. Results are from 1 of 2 representative experiments.

tion into the lateral tail vein. Larger doses were divided (≤1 mg/kg/dose) and given 4 h apart. lAmB (AmBisome; gift of Vestar, San Dimas, CA) was reconstituted according to the manufacturer's recommendations. Stock solutions were stored at 4°C up to 1 week. For iv injection, the lAmB was diluted in 5% dextrose to the required concentrations and given iv in a single daily dosage. Treatment was started 6 h after challenge and was continued until sacrifice.

Evaluation of the effect of antimycotic regimens. The animals were sacrificed by exposure to CO_2, and the yeast infection was quantitated by culturing serial dilutions of homogenates, which were prepared from subcutaneous pouches and parenchymatous organs by individual homogenization with Teflon pestles as described previously [25].

In vitro comparison of the fungicidal activity of AmB formulations. For in vitro studies, an overnight culture of test strains grown in TSB were washed in MEM (Life Technologies GIBCO BRL, Basel, Switzerland) and visually adjusted to a concentration of 10^6 yeast cells/mL before exposure to graded concentrations (0–50 mg/L) of AmB-d and lAmB. After incubation for 24 h in a 5% CO_2 atmosphere, yeast cells were washed three times in TSB, and serial dilutions were cultured in duplicate on TSA for quantification of colony-forming units.

Table 1. Fungicidal activity of amphotericin B–desoxycholate (AmB-d) and liposomal amphotericin B (lAmB) against 4 isolates of *Candida albicans* in vitro.

Strain	AmB-d	lAmB	Ratio of lAmB to AmB-d
SD1	0.67	3.4	5.0
NC31	0.76	6.9	9.1
NC19	0.75	2.9	3.9
SN35	0.35	2.9	8.3

NOTE. Data are concentration (mg/L) required to reduce initial inoculum (10^6 cfu) of yeast cells by 2 logs (99% killing).

Table 2. Toxicity of amphotericin B–desoxycholate (AmB-d) and liposomal amphotericin B (lAmB) given to immunosuppressed mice daily for 6 days.

Dose of AmB (mg/kg)	AmB-d*	lAmB
8	ND	0/8
4	ND	0/8
3	8/8	0/8
2	0/8	0/8
1	0/8	ND

NOTE. Data are no. mice dead/no. inoculated. ND, not done.
* Given as fractionated doses (≤1 mg/kg) 4 h apart. With this regimen, mice receiving 3 mg/kg/day died within 36 to 72 h of first dose.

Statistical analysis. Results are given as mean ± SD from 3–5 animals per group and time point. Values were compared by Student's *t* test.

Results

Comparison of the fungicidal activity of AmB-d and lAmB in vitro. At concentrations of 0.2 and 0.8 mg/L, respectively, AmB-d and lAmB were fungistatic; 99% killing was seen at concentrations <0.4 mg/L AmB-d and at 3.2 mg/L lAmB. Both results show that lAmB is four to eight times less active than AmB-d in vitro (figure 1). Comparable results were obtained with other *C. albicans* isolates (table 1) and when the technique was modified by placing test tubes on a rotary shaker for agitation during exposure of fungus cells to AmB (data not shown).

Toxicity of AmB-d and lAmB in mice. In preparation of the therapeutic in vivo studies, the toxicity of both AmB formulations was compared in mice receiving the immunosuppressive cortisone regimen (see Materials and Methods).

Table 3. Change in colony-forming units of 2 strains of *Candida albicans* in pneumatized pouches of immunosuppressed mice after 3-day treatment with amphotericin B–desoxycholate (AmB-d) or liposomal amphotericin B (lAmB).

AmB-d (mg/kg/day)	Change (log cfu)	lAmB (mg/kg/day)	Change (log cfu)
C. albicans SD1			
None	+0.94 (±0.06)	2	+0.53 (±0.42)
1	−0.21 (±0.39)	4	0.05 (±0.34)
2	−0.68 (±0.43)*	8	−0.30 (±0.40)
C. albicans NC19			
None	+1.01 (±0.41)	2	−0.06 (±0.36)
1	−0.79 (±0.36)	4	−0.06 (±0.26)
2	−1.53 (±0.46)*	8	−1.42 (±0.44)

NOTE. Treatment was started 6 h after challenge with 10^6 yeast cells and was followed by further doses 30 and 54 h after challenge. Mice were sacrificed 24 h after last therapeutic dose. Mean ± SD from 3–5 mice/group.
* Antifungal activity was significantly higher than that of 4 mg of lAmB ($P < .01$).

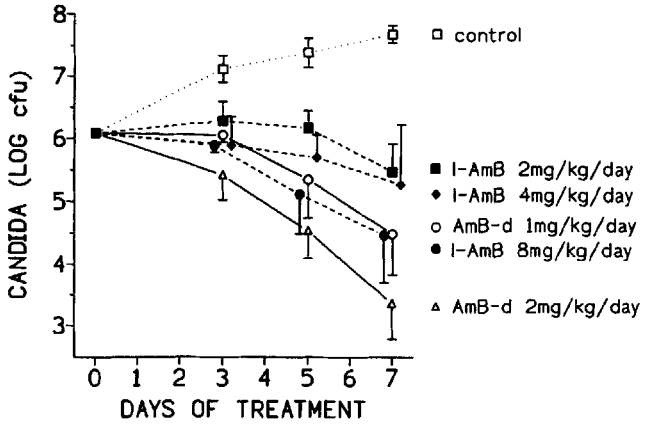

Figure 2. Antifungal activity of amphotericin B–desoxycholate (AmB-d) and liposomal amphotericin B (l-AmB) in pneumatized subcutaneous pouches. After immunosuppression with 5 mg of cortisone twice, 1.2×10^6 cfu of *Candida albicans* SD1 was inoculated into pouches. Mean ± SD from 4 mice per group and time point.

Injections of graded doses (milligrams per kilogram) of AmB (active drug) were given. Mice died within 90 min of injection if given >1 mg of AmB-d in an unfractionated dose; therefore, higher daily doses were divided into amounts ≤1 mg/kg and given 4 h apart. With this regimen, the mice receiving 3 mg/kg/day died 36–72 h after the first injection. Mice tolerated much higher doses of lAmB than AmB-d. The highest tolerated dosage of AmB-d was 2 mg/kg/day (table 2).

Comparison of AmB-d and lAmB in a model of local candidiasis. In a first series of experiments with 2 strains of *C. albicans*, AmB-d given for 3 days was four to eight times more active against *C. albicans* on a dose per weight basis than was lAmB (table 3). Treatment for 7 days after inoculation of 1.2×10^6 cfu of *C. albicans* into subcutaneous pouches further widened the discrepancy between the antifungal activity of the two formulations. In all groups of mice, all doses studied resulted in a reduction of colony-forming units by day 7, which was also 7 days after the last immunosuppression with cortisone acetate. In addition, 2 mg/kg AmB-d proved more active than 8 mg/kg lAmB ($P = .0594$), and 1 mg/kg AmB-d had an effect comparable to 8 mg/kg lAmB (figure 2). Thus, lAmB appeared about four to eight times less potent than AmB-d in the local model of candidiasis.

Comparison of AmB-d and lAmB in a model of systemic candidiasis. In the systemic model, 1 mg/kg AmB-d was more active than 2 and 4 mg/kg lAmB against *C. albicans* in the kidneys ($P = .0013$; figure 3A). After a higher inoculum of *C. albicans* (see figure 3B), considerably larger numbers of colony-forming units were recovered from the kidneys. At 0.5 mg/kg, AmB-d showed antifungal activity comparable to 4 mg/kg lAmB. For the higher doses of AmB-d (1 and 2 mg/kg), the ratio of its antifungal potency compared with lAmB was ~1:8 (figure 3B).

In all experiments with the systemic model of candidiasis, a much lower number of fungi was recovered from the livers than the kidneys because *C. albicans* was rapidly cleared from this organ despite immunosuppression with cortisone. Therefore, a comparison of the therapeutic activity of the two formulations was only possible on day 1, which was 1 day after challenge and after only 1 dose of AmB. Treatment with different concentrations of AmB-d suggested a dose-effect relationship, and even the lowest dose (0.5 mg/kg) of AmB-d had antifungal activity. lAmB at doses of 4–16 mg/kg was not more active than 0.5 mg/kg AmB-d, and no definitive dose response was obtained for lAmB (table 4).

Discussion

These studies show that lAmB, while considerably less toxic, is four to eight times less active than AmB-d in vitro

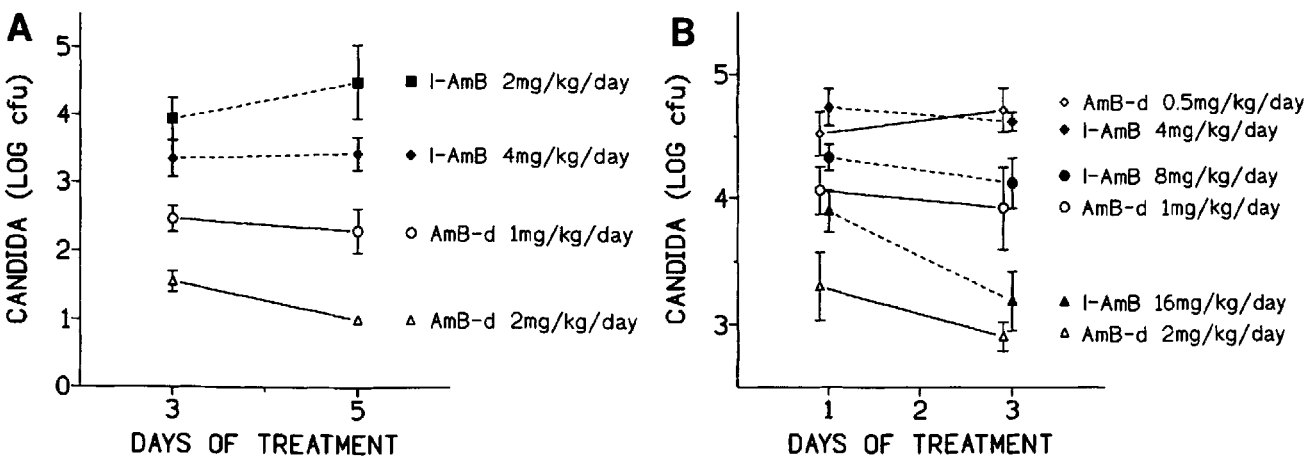

Figure 3. Antifungal activity of amphotericin B–desoxycholate (AmB-d) and liposomal amphotericin B (l-AmB) in kidneys of immunosuppressed mice challenged intravenously with 10^4 cfu (**A**) or 7.5×10^5 cfu (**B**) of *Candida albicans* SD1. Mean ± SD from 4 mice per group and time point.

Table 4. Number of colony-forming units of *Candida albicans* SD1 after 1 day of treatment with amphotericin B–desoxycholate (AmB-d) or liposomal amphotericin B (lAmB) in livers of immunosuppressed mice.

AmB-d (mg/kg)	Log cfu	lAmB (mg/kg)	Log cfu
None	4.67 (±0.06)	None	4.67 (±0.06)
0.5	3.05 (±0.25)	4.0	2.94 (±0.38)
1.0	2.89 (±0.18)	8.0	2.82 (±0.43)
2.0	2.59 (±0.19)	16.0	2.87 (±0.48)

NOTE. Intravenous challenge dose was 7.5×10^5 cfu. Mean ± SD from 4 mice/group.

and in vivo. Not surprisingly, a localized infection with *C. albicans* in subcutaneous pouches was difficult to treat, and compared with the treatment of systemic candidiasis, it required more and higher doses of AmB for an adequate therapeutic effect. Surprisingly, however, there was no difference between the ratio of activity of AmB-d and lAmB in the models of localized or systemic candidiasis. The observation that lAmB was less active both in vitro and in the animal models indicates that the less toxic polyene antifungal drug entrapped in liposomes is also therapeutically less active. Therefore, we must question whether the therapeutic index of the lAmB preparation studied here is indeed increased over that of AmB-d.

Previous observations on the relative activity of liposomal and free AmB in vitro remain controversial: Several investigators have found an equivalent activity [26, 27], while others have found a significantly lower activity [28–32] for lAmB. Most in vivo studies indicate that lAmB, while less toxic than AmB-d, is also less active at equal doses [4, 33–37]. It has, however, been claimed in these studies, on the basis of acute toxicity of rapidly injected AmB-d in small laboratory animals, that the therapeutic index for lAmB is better than for AmB-d. These studies do not, however, consider that AmB-d is not given clinically as an iv bolus. In this and previous [38] studies, mice tolerated much higher AmB-d doses if the drug was given in multiple fractions. In mice, the activity of the maximally tolerated dosage (2 mg/kg/day) of AmB-d could not be reached by 8–16 mg/kg/day lAmB.

In conclusion, the lAmB used here was less toxic than AmB-d, but it was also considerably less active. Therefore, the therapeutic index of lAmB and possibly other new formulations of AmB should be thoroughly evaluated before a widespread clinical use of lAmB can be recommended. The data also suggest that in such studies, lAmB should be tested at higher doses than the usual doses of AmB-d.

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